# Dioxinlike Components in Incinerator Fly Ash: A Comparison between Chemical Analysis Data and Results from a Cell Culture Bioassay

**Markus Till,[1] Peter Behnisch,[2] Hanspaul Hagenmaier,[2] Karl Walter Bock,[1] and Dieter Schrenk[3]**

[1]Institute of Toxicology; and [2]Institute of Organic Chemistry, University of Tübingen, Tübingen, Germany; [3]Food Chemistry/Environmental Toxicology, University of Kaiserslautern, Kaiserslautern, Germany

Potent polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxinlike polychlorinated biphenyls (PCBs) are among the most relevant toxic emissions from incinerators. Induction of cytochrome P450 1A1-catalyzed 7-ethoxyresorufin *O*-deethylase (EROD) activity in mammalian cell culture (EROD bioassay) is thought to be a selective and sensitive parameter used for the quantification of dioxinlike compounds. Fly ash extracts from municipal waste incinerators (MWI), a crematorium, wood combustors, and a noble metal recycling facility were analyzed in the EROD bioassay using rat hepatocytes in primary culture. Fractions containing 2,3,7,8-substituted PCDDs/PCDFs, dioxinlike PCBs, and 16 major polycyclic aromatic hydrocarbons (PAHs) were isolated from the extract and analyzed by gas chromatography-mass spectrometry (GC-MS) and by the EROD bioassay. It was found that with MWI samples the bioassay of the extract resulted in a two- to fivefold higher estimate of TCDD equivalents (TEQ) than the chemical analysis of PCDDs/PCDFs and PCBs. However, the outcome of both methods was significantly correlated, making the bioassay useful as a rough estimate for the sum of potent PCDDs/PCDFs and dioxinlike PCBs in extracts from MWI fly ash samples and in a fly ash sample from a crematorium. In noble metal recycling facility and wood combustor samples, higher amounts of PAHs were found, contributing to more pronounced differences between the results of both methods. The remaining unexplained inducing potency in fly ash samples probably results from additional dioxinlike components including certain PAHs not analyzed in this study. The hypothesis that emissions from MWI of hitherto unidentified dioxinlike compounds are higher by orders of magnitude than emissions of potent PCDDs/PCDFs and dioxinlike PCBs could not be confirmed. We found no indication for a marked synergistic interaction of dioxinlike fly ash components in the bioassay. *Key words:* bioassay, dioxin, EROD, fly ash, incineration, PAH, PCB.

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The incineration of municipal waste, wood, and other organic material is a major source of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), and probably also of polychlorinated biphenyls (PCBs), in the environment (*1–4*). A number of congeners of PCDDs, PCDFs, or PCBs lead to pronounced toxic effects in experimental animals, described as dioxinlike effects because they resemble those exerted by the most toxic compound in this group, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). These effects include immunotoxicity, teratogenicity, impairment of hormone function, and tumor promotion (*5–7*). Because individual PCDDs and PCDFs can differ in their toxic potency by several orders of magnitude, the use of equivalency factors for the risk estimation of mixtures of these compounds has been widely accepted in toxicology. This method is based on the chemical analysis of the most potent, 2,3,7,8-substituted PCDDs and PCDFs, and multiplication of their concentrations with published international toxic or TCDD equivalency factors (I-TEFs) (*8*). Following a suggestion by a World Health Organization (WHO) working group (*9*), a number of dioxinlike PCBs congeners have also been attributed with equivalency factors (WHO-TEFs). The total amount of components, e.g., in an incinerator fly ash, attributed with equivalency factors is expressed as toxic or TCDD equivalents (TEQ) per mass unit. In various countries, the emissions of incinerators are regulated on the basis of TEQ per gas volume emitted from the plant as calculated from the chemical analysis of 2,3,7,8-substituted PCDDs/PCDFs.

The concept of I-TEFs is based on the assumption that the dioxinlike effects of the individual congeners are additive. This requirement was not strictly fulfilled in all experiments with mixtures reported so far, with some showing synergistic or antagonistic interactions (*7,10–12*). However, the fact that most if not all toxic effects of TCDD and related compounds are mediated by a common receptor, the dioxin or aryl hydrocarbon receptor (AhR), is in favor of the concept of additivity (*5,7,13*). Induction of cytochrome P450 (CYP)1A1 is one of the best understood and most sensitive biochemical effects mediated via the ligand-activated AhR (*3,14,15*). In fact, a very good correlation was found between the rank order of PCDF congeners relating their *in vivo* toxicity in rats to their CYP1A1-inducing potency (*6*). These findings and the common biochemical mode of action of dioxinlike compounds led to the establishment of bioassays based on AhR activation. Among these, induction of CYP1A1 in mammalian cell culture is the most widely used (*16–18*). In particular, additive or almost additive interaction was found for the induction of CYP1A1-catalyzed 7-ethoxyresorufin *O*-deethylase (EROD) activity in rat hepatocytes in primary culture, in rat H4IIE hepatoma cells, or in human HepG2 hepatoma cells, using complex PCDD or PCB mixtures (*19–23*) or extracts from environmental samples (*24*). Moreover, EROD-TEF values of PCDDs (*19*) and PCBs (*21*) in the bioassay were in very good agreement with I-TEFs and WHO-TEFs, respectively.

CYP1A1-related bioassays have been used in previous studies (*25–28*) for the biological evaluation of incinerator fly ash. However, the contribution of AhR agonists other than PCDDs/PCDFs was not determined. In fact, the outcome of the EROD bioassay may be influenced by PCBs and other receptor agonists such as polycyclic aromatic hydrocarbons (PAHs) present in fly ash. Chemical analysis of fly ash is usually not designed to detect these and other AhR agonists such as polybrominated dibenzo-*p*-dioxins, dibenzofurans, or biphenyls and polyhalogenated naphthalenes and biphenyl ethers, etc. The inducing effect of both identified and unknown AhR agonists are, in principle, detected in the EROD bioassay.

In the present investigation, we analyzed fly ash samples from municipal waste incinerators (MWI), a crematorium, wood combustors (WCB), and a noble metal recycling facility (NMRF) for potent

Address correspondence to D. Schrenk, Food Chemistry/Environmental Toxicology, University of Kaiserslautern, Erwin-Schroedinger-Strasse, D-67663 Kaiserslautern, Germany.

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PCDDs/PCDFs and for dioxinlike PCBs. Furthermore, a number of major PAHs was also analyzed. In parallel experiments using the EROD bioassay in rat hepatocyte culture, we found that the EROD-TEQ values in many instances were about two to five times higher than TEQ calculated from the chemical analysis (chemical TEQ). Evidence is provided that PAHs and unidentified components with dioxinlike activity in the bioassay may contribute to this difference.

## Methods

***Sample Extraction and Chemical Analysis.*** $^{13}C$-labeled PCDD/PCDF and PCB standards were purchased from Promochem (Wesel, Germany). All solvents used were nanograde.

Fly ash samples were obtained from an electrostatic precipitator in the municipal waste incinerator in Stuttgart-Münster (boiler 27-29); from a dioxin disengager of a noble metal recycling facility in Pforzheim before (NMRF 1a and 1b) and after (NMRF 1c) Sorbalit (a limestone/charcoal-adsorbent; Märker, Harburg, Germany) addition into the flue gas; from the crematorium at the Prag-graveyard in Stuttgart, and from the municipal waste incinerator in Ulm-Weißenhorn after Sorbalit injection into the flue gas (bag house dust). The fly ash samples from wood combustors were obtained from commercial wood burning facilities in Stuttgart. All samples were collected between 1994 and 1996.

Sample preparation, extraction, and analysis used for PCDDs, PCDFs, PAHs, and PCBs included spiking with $^{13}C$-labeled (PCBs, PCDDs/PCDFs) or deuterated (PAHs) internal standards, Soxhlet extraction using toluene, and chromatographic cleanup and separation in PCDD/PCDF-, PAH-, and PCB-containing fractions.

Final analysis was carried out using an HP 5970 A mass selective detector (Hewlett-Packard, Böblingen, Germany) interfaced directly with an HP 5890 A gas chromatograph (Hewlett-Packard) for the analysis of PCBs and PCDDs/PCDFs and an HP 5890/HP 5972 gas chromatograph-mass spectrometer (GC-MS) for the PAH analyses. The following PAHs were analyzed: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a,h*]anthracene, benzo[*g,h,i*]perylene, and indeno[1,2,3,*c,d*]-pyrene.

Extracts were prepared as follows: 50–100 g fly ash was mixed with 300 ml 1 N hydrochloric acid and stirred for 1 hr. After filtration the residue was air dried. The acid-treated fly ash was extracted with toluene in a Soxhlet extractor for 20 hr.

An aliquot (25%) of the various extracts was used for the EROD bioassay. Another aliquot (25%) was spiked with $^{13}C$-labeled PCB standards (IUPAC numbers 3, 15, 28, 52, 77, 101, 105, 118, 126, 138, 153, 156, 169, 180, 202, 209) and with a $^{13}C$-labeled PCDD/PCDF standard mixture containing all 2,3,7,8-substituted congeners. The solvent was evaporated to dryness and the extract redissolved in 100–200 ml *n*-heptane. After addition of 20–50 g silica gel (ICN silica 63–300 µm, active; ICN Biochemicals, Eschwege, Germany)/44% (w/w) concentrated sulfuric acid (95–97%, for analyses; Merck, Darmstadt, Germany), the extract was treated for 10 min at 70°C. The heat-treated extract was reduced to about 0.5–3 ml. The sample was placed on top of a column (2×30 cm) filled with (bottom to top) 25 g Alumina B Super I (ICN Biochemicals) and 3 g silica gel/44 % concentrated sulfuric acid. The column was eluted with 350 ml *n*-heptane/dichloromethane (1:1). After the evaporation of the solvent to about 1 ml, the cleanup was continued with a mini column with 0.8 g Alumina B Super I and 0.3 g silica gel/44% concentrated sulfuric acid. After preelution with 4 ml *n*-pentane, the PCBs fraction was collected by elution with 6 ml *n*-heptane/dichloromethane (98:2), followed by eluting the non-*ortho*-chlorinated PCBs with 1.8 ml benzene and the PCDD/PCDF fraction with 6 ml *n*-heptane/dichloromethane (1:1).

Analysis was performed by high resolution gas chromatography (GC)/low resolution mass spectrometry (MS) using a 15-m DBXLB or a 30-m DB 5 column (both from J & W Scientific, Folsom, CA) for the PCBs and the $Cl_7/Cl_8$ PCDDs/PCDFs, and a CP-Sil 88 (Chrompack, Frankfurt, Germany) column for the $Cl_4$ to $Cl_6$ PCDDs/PCDFs. The DBXLB column had the advantage of separating the PCB pairs of IUPAC-Numbers 28/31, 118/124, and 156/157. The mass spectrometer was run in the SIM (selected ion monitoring) mode.

For analysis of PAHs, an aliquot of the extract corresponding to 0.08–0.3 g sample was spiked with a deuterated standard mixture and the resulting mixture applied to a column (0.7×14 cm) filled with 2.5 g basic Alumina B Super I (mixed with 10% water); PAHs were eluted with 10 ml toluene/cyclohexane (1:1) and analyzed with GC-MS.

***Preparation of PCDD/PCDF-, PCB-, and PAH-enriched fractions.*** Another aliquot of the extract was used to obtain fractions containing PCDDs/PCDFs, PCBs, and PAHs, which could also be analyzed in the EROD bioassay. In this case, no labeled standards could be added. To carry out a chemical analysis of these fractions, an aliquot containing the labeled standards was worked up in parallel. The extract was applied to a column filled with 2.5 g basic Alumina B Super I (mixed with 10% water), and PCBs, PCDDs/PCDFs, and PAHs were eluted with 20 ml toluene/cyclohexane (1:1). After evaporation of the solvent, the sample was dissolved in 200 µl toluene and injected in an HPLC system (Merck 655A-11) with a 19×250 mm column filled with Nucleosil-5-$NO_2$ (Macherey-Nagel GmbH, Düren, Germany). The first PAH fraction was eluted with 9.5 ml *n*-heptane; PCBs and PCDDs/PCDFs were eluted with 21 ml *n*-heptane and, finally, the main PAH fraction was eluted with 45 ml *n*-heptane. After equilibration with 30 ml *n*-heptane, 100 ml acetone, and another 30 ml *n*-heptane, the next sample was injected. The PAH fractions were combined, analyzed by GC-MS, and prepared for the EROD bioassay.

The PCB/PCDD/PCDF fractions were cleaned up further by heat treatment with concentrated sulfuric acid at 70°C and subsequent chromatography on a minicolumn filled with Alumina B Super I (0.8 g). The mono- and di-*ortho*-substituted PCBs were eluted with 6 ml heptane/dichloromethane (98:2), the non-*ortho*-substituted PCBs with 1.8 ml benzene, and the PCDDs/PCDFs with 6 ml *n*-heptane/dichloromethane (1:1). After drying by a gentle nitrogen stream to about 10–30 µl, the PCBs and PCDDs/PCDFs were analyzed by GC-MS.

***Cell Culture Bioassay.*** Dulbecco's Modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Biochrom (Berlin, Germany), calf serum and fetal calf serum from Gibco (Paisley, UK), collagenase type I from Sigma (St. Louis, MO), and isocitrate dehydrogenase from Boehringer (Mannheim, Germany).

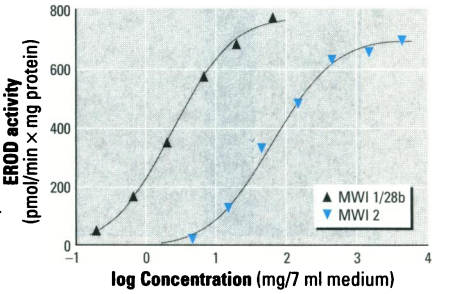


**Figure 1.** Log-probit curves of 7-ethoxyresorufin *O*-deethylase (EROD) induction in rat hepatocytes in primary culture using fly ash extracts from sample municipal waste incinerator (MWI) 1/28b and MWI 2. The data represent means of four independent experiments.

Hepatocytes were isolated from adult male Wistar rats (Savo, Kisslegg, Germany) kept on standard diet (Altromin, Lage, Germany) and tap water. After anesthesia with sodium pentobarbital (100 mg/kg body weight, given by intraperitoneal injection), livers were perfused with sterile calcium-free, EGTA-supplemented buffer (6.3 g/l NaCl; 0.32 g/l KCl; 0.27 g/l $MgSO_4 \times 7\ H_2O$; 0.15 g/l $KH_2PO_4$; 1.81 g/l $NaHCO_3$; 3.58 g/l HEPES; 1.5 g/l D-glucose $\times\ H_2O$; 0.038 g/l EGTA; pH 7.2) for 10 min. Livers were then perfused with sterile calcium-containing buffer (6.3 g/l NaCl; 0.32 g/l KCl; 0.27 g/l $MgSO_4 \times 7\ H_2O$; 0.15 g/l $KH_2PO_4$; 1.81 g/l $NaHCO_3$; 3.58 g/l HEPES; 1.5 g/l D-glucose $\times\ H_2O$; 0.58 g/l $CaCl_2 \times H_2O$; pH 7.2) supplemented with 90 mg collagenase/ 200 ml. Hepatocytes were then dispersed in washing buffer (6.3 g/l NaCl; 0.32 g/l KCl; 0.27 g/l $MgSO_4 \times 7\ H_2O$; 0.15 g/l $KH_2PO_4$; 1.81 g/l $NaHCO_3$; 3.58 g/l HEPES; 1.5 g/l D-glucose $\times\ H_2O$; 0.58 g/l $CaCl_2 \times H_2O$, 20.0 g/l bovine serum albumin; pH 7.2), filtered through sterile silk gauze, and washed two times with washing buffer. Viability was estimated using trypan blue exclusion, and preparations showing viabilty >85% were used for cell culture.

Hepatocytes were plated at a density of 100,000 cells/$cm^2$ on collagen-coated petri

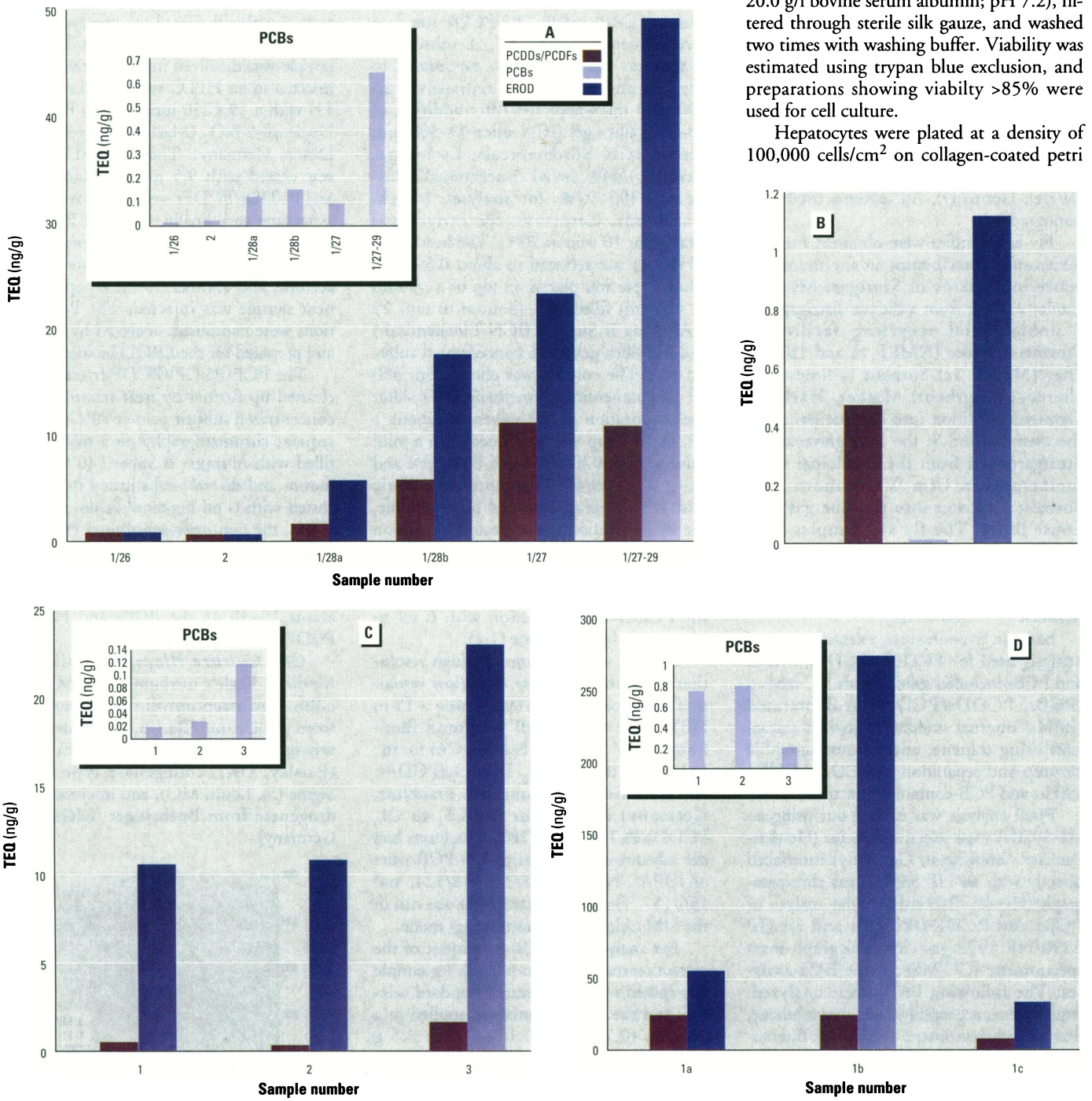


**Figure 2.** Levels of potent PCDDs/PCDFs [in nanograms international toxic equivalents (I-TEQ)/g] and dioxinlike polychlorinated biphenyls (PCBs) [in nanograms World Health Organizaton toxic equivalents (WHO-TEQ)/g] in comparison to 7-ethoxyresorufin *O*-deethylase (EROD)-TEQ, and the sum of 16 major PAHs (µg/g) in extracts of fly ash samples from (A) municipal waste incinerators (MWI); (B) a crematorium; (C) wood combustors (WCB); and (D) a noble metal recycling facility (NMRF). Insets show PCB levels too low to be seen on other scales. Fly ash extracts were prepared, and potent PCDDs/PCDFs, dioxinlike PCBs, and 16 major PAHs were analyzed as described in Methods. EROD-TEQ values represent the means of four independent determinations using the median effective concentration of EROD induction in rat hepatocytes in primary culture.

dishes (9 cm diameter) in DMEM supplemented with 10% calf serum, 10% fetal calf serum, 0.1 M dexamethasone, 100 U/ml penicillin, and 100 g/ml streptomycin as described (*19*). After 2 hr, medium was replaced by fresh medium, fly ash extracts or TCDD were added in DMSO (0.5 % final concentration), and cells were harvested after 48 hr. Cells were washed two times with saline and harvested by scraping off with cold 0.2 M sucrose/0.05 M Tris-HCl, pH 7.4 (buffered sucrose). The cells were then centrifuged for 10 min at 1000×*g* and homogenized with a Dounce homogenizer in buffered sucrose on ice. EROD activity was determined according to Burke and Mayer (*29*). Protein was analyzed in homogenates using bovine serum albumin as a standard (*30*). Dose–response curves were calculated using a computerized log-probit procedure (SAS Institute, Cary, NC; Technical report P-179), which also allows calculation of median effective concentration ($EC_{50}$) values and 95% confidence intervals.

## Results

CYP1A1-catalyzed EROD activity in rat hepatocyte cultures was inducible with extracts from all fly ash samples investigated. Fitting of a log-probit function to the mean values from four independent experiments led to sigmoidal concentration–response curves as examplified in Figure 1 for two fly ash extracts of MWI samples. Using TCDD as inducer in a reference experiment (not shown), the EROD-TEQ per gram fly ash were then calculated based on comparison of effective concentration values. Chemical analysis of the extracts from MWI fly ash samples showed that the content of PCDDs/PCDFs and PCBs varied considerably between samples ranging from 0.44 to 11.2 ng TEQ/g fly ash for PCDDs/PCDFs and from 0.007 to 0.64 ng TEQ/g fly ash for dioxinlike PCBs (Fig. 2A, Table 1).The dioxinlike PCBs accounted for about 1% of total calculated TEQ. In most instances, EROD-TEQ values determined in the bioassay were higher by a factor of 2–5 than expected from the chemical analysis of PCDDs/PCDFs and PCBs.

Further chemical analysis revealed that PAHs were also present in MWI samples and in all other samples analyzed. The difference between EROD-TEQ and chemical TEQ was found to be roughly correlated to the PAH content (sum of 16 PAHs) analyzed in MWI samples (Fig. 3). A more detailed analysis (Table 2), however, did not reveal a clear-cut correlation between the PAH pattern and the EROD-TEQ/chemical TEQ difference.

In a fly ash sample from a crematorium (Fig. 2B), PCDDs/PCDFs, dioxinlike PCBs, and PAHs could be identified. Total TEQ were in the same range as in the MWI sample with the lowest contamination. An approximately twofold higher TEQ-EROD was found than expected from the chemical analysis of PCDDs/PCDFs and dioxinlike PCBs. The pattern of PAHs resembled those of MWI fly ash samples.

In fly ash samples from wood combustors (Fig. 2C, Table 1), chemical TEQ contents were in the range of MWI fly ash samples with lower contamination. The contribution of PCBs to the total chemical TEQ value was clearly lower than in the case of MWI samples (<0.5%). In contrast, the relative content of PAHs (compared to PCDDs/PCDFs) was higher than in MWI samples. In the three WCB samples analyzed, no correlation was found between total PAH content and the gap between EROD-TEQ and chemical TEQ. In general, WCB fly ash samples contained higher relative amounts of highly condensed PAHs such as benzo[*b*]fluoranthene or benzo[*a*]pyrene, e.g., in sample WCB3 showing the highest EROD-TEQ/chemical TEQ difference among these samples.

TEQ-values similar to those in strongly contaminated MWI samples were found in

**Table 1.** Levels of compounds in fly ash samples

| | PCDDs/PCDFs (ng I-TEQ/g) | PCBs (ng WHO-TEQ/g) | EROD (ng TEQ/g) | PAH content (µg/g) | ΔEROD-chemical TEQ (ng/g) |
|---|---|---|---|---|---|
| Municipal waste incinerator | | | | | |
| 1/26 | 0.44 | 0.01 | 0.72 | 0.05 | 0.28 |
| 2 | 0.67 | 0.02 | 0.66 | 0.19 | –0.03 |
| 1/28a | 1.61 | 0.12 | 5.66 | 0.52 | 3.94 |
| 1/28b | 5.69 | 0.15 | 17.74 | 0.99 | 11.91 |
| 1/27 | 11.20 | 0.09 | 23.38 | 0.30 | 12.09 |
| 1/27-29 | 10.90 | 0.64 | 49.47 | 0.86 | 37.93 |
| Crematorium | 0.47 | 0.01 | 1.12 | 2.85 | 0.64 |
| Wood combustors | | | | | |
| 1 | 0.45 | 0.02 | 10.37 | 7.40 | 9.9 |
| 2 | 0.25 | 0.03 | 10.93 | 59.64 | 10.62 |
| 3 | 1.74 | 0.12 | 23.31 | 1.91 | 21.45 |
| Noble metal recycling facility | | | | | |
| 1a | 24.9 | 0.74 | 57.09 | 301.9 | 31.45 |
| 1b | 25.2 | 0.79 | 279.22 | 536.4 | 253.23 |
| **1c** | **12.8** | **0.21** | **34.63** | **0.7** | **21.62** |

Abbreviations: PCDDs/PCDFs, polychlorinated dibenzodioxins and polychlorinated dibenzofurans; PCBs, polychlorinated biphenyls; PAH, polycyclic aromatic hydrocarbon; EROD, 7-ethoxyresorufin *O*-deethylase; TEQ, toxic equivalents; I, international; WHO, World Health Organization.

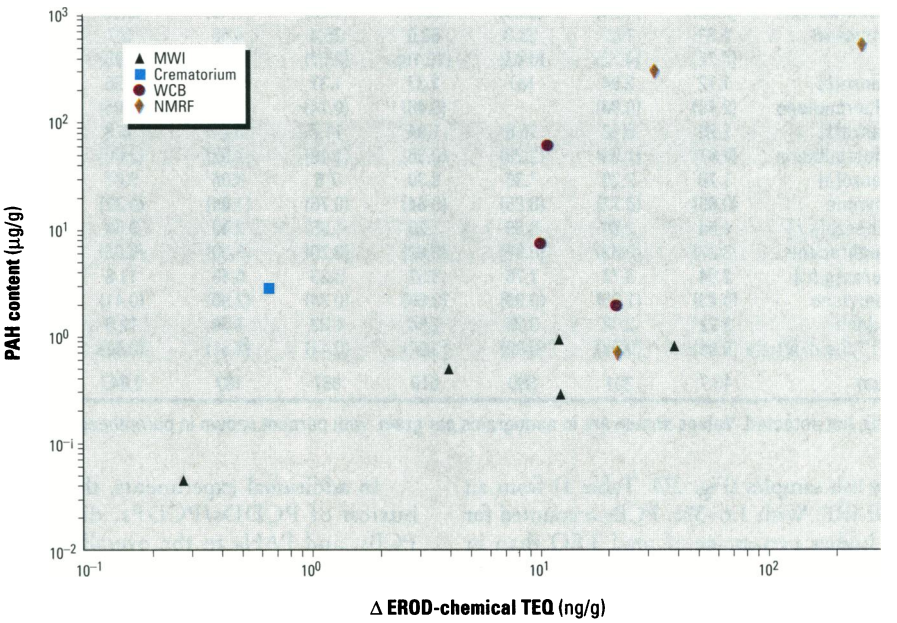


**Figure 3.** Correlation between the sum of 16 major polycyclic aromatic hydrocarbons (PAHs) and the difference between chemical toxic equivalents (TEQ) and 7-ethoxyresorufin *O*-deethylase (EROD)-TEQ in extracts of fly ash samples from municipal waste incinerators (MWI), a crematorium, wood combustors (WCB), and a noble metal recycling facility (NMRF).

**Table 2.** Difference between chemical toxic equivalents (TEQ) and 7-ethoxyresorufin *O*-deethylase (EROD)-TEQ, and chemical analysis of 16 major polycyclic aromatic hydrocarbons (PAHs) in extracts of fly ash samples from municipal waste incinerators (MWI), a crematorium, wood combustors (WCB), and a noble metal recycling facility (NMRF).

| PAHs | MWI 1/26 | MWI 1/27 | MWI 1/27-29 | MWI 1/28a | MWI 1/28b | MWI 2 | Crematorium | WCB 1 | WCB 2 | WCB 3 | NMRF 1a | NMRF 1b | NMRF 1c |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Difference (TEQ ng/g) | 0.28 | 12.09 | 37.93 | 3.94 | 11.91 | – | 0.64 | 9.90 | 10.62 | 21.46 | 31.45 | 253.24 | 21.62 |
| Naphthalene | 8.46 (18.51) | ND | ND | 21.0 (4.05) | 222 (22.49) | 58.0 (31.02) | 927 (32.56) | ND | ND | ND | 846 (0.28) | 10,200 (1.90) | ND |
| Acenaphthylene | ND | ND | 52.5 (6.10) | 2.24 (0.43) | 11.4 (1.16) | 1.70 (0.91) | 72.2 (2.54) | 8.42 (0.11) | 1,473 (2.47) | 46.3 (2.42) | 483 (0.16) | 1,500 (0.28) | 0.14 (0.02) |
| Acenaphthene | 2.35 (5.14) | ND | 19.2 (2.23) | 22.5 (4.34) | 4.77 (0.48) | 2.2 (1.18) | 59.0 (2.07) | ND | 156 (0.26) | ND | 5.1 (0.00) | ND | ND |
| Fluorene | 0.93 (2.04) | 49.8 (16.54) | 23.5 (2.73) | 4.21 (0.81) | 21.4 (2.17) | 3.4 (1.82) | 28.5 (1.00) | ND | 11.4 (0.02) | 23.7 (1.24) | 41.8 (0.01) | 31.3 (0.01) | ND |
| Phenanthrene | 9.11 (19.93) | 144 (47.84) | 418 (48.60) | 300 (57.92) | 218 (22.09) | 25.0 (13.37) | 780 (27.40) | 1,810 (24.45) | 8,830 (14.81) | 258 (13.49) | 4,620 (1.53) | 24,000 (4.47) | 60.0 (8.53) |
| Anthracene | 6.73 (14.73) | 25.4 (8.44) | 30.8 (3.58) | 75.0 (14.48) | 334 (33.84) | 40.3 (21.55) | 83.7 (2.94) | 242 (3.27) | 979 (1.64) | ND | ND | 6,670 (1.24) | ND |
| Fluoranthene | 1.93 (4.22) | 27.4 (9.10) | 123 (14.30) | 12.7 (2.45) | 77.2 (7.82) | 16.0 (8.56) | 344 (12.08) | 1,650 (22.29) | 10,000 (16.77) | 292 (15.27) | 34,500 (11.43) | 33,600 (6.26) | 101 (14.37) |
| Pyrene | 1.31 (2.87) | 16.3 (5.42) | 71.0 (8.26) | 8.24 (1.59) | 31.1 (3.15) | 11.3 (6.04) | 286 (10.05) | 1,120 (15.13) | 11,000 (18.44) | 137 (7.17) | 34,500 (11.43) | 29,200 (5.44) | 11.9 (1.69) |
| Benzo[*a*]-anthracene | 1.17 (2.56) | 5.36 (1.78) | 22.8 (2.65) | 2.29 (0.44) | 11.8 (1.20) | 2.68 (1.43) | 67.8 (2.38) | 380 (5.13) | 2,730 (4.58) | 244 (12.76) | 6,890 (2.28) | 7,970 (1.49) | ND |
| Chrysene | 1.93 (4.22) | 14.2 (4.72) | 55.3 (6.43) | 52.6 (10.15) | 25.4 (2.57) | 4.56 (2.44) | 107 (3.76) | 631 (8.52) | 3,667 (6.15) | 258 (13.49) | 43,700 (14.48) | 12,730 (2.37) | 197 (28.02) |
| Benzo[*b*]-fluoranthene | 1.12 (2.45) | 2.64 (0.88) | ND | 2.37 (0.46) | 1.37 (0.14) | 1.85 (0.99) | 1.56 (0.05) | ND | ND | 278 (14.54) | 9,090 (3.01) | 51,600 (9.62) | ND |
| Benzo[*k*] fluoranthene | 2.20 (4.81) | 5.10 (1.69) | 19.6 (2.28) | 1.84 (0.36) | 11.7 (1.19) | 3.84 (1.52) | 56.8 (2.00) | 657 (8.87) | 3,950 (6.62) | ND | 21,900 (7.25) | 67,080 (12.50) | 322 (45.80) |
| Benzo[*a*] pyrene | 1.76 (3.85) | 2.20 (0.73) | 7.35 (0.85) | 3.30 (0.64) | 7.5 (0.76) | 3.06 (1.64) | 9.07 (0.32) | 202 (2.73) | 4,080 (6.84) | 301 (15.74) | 27,300 (9.04) | 44,720 (8.34) | 4.4 (0.63) |
| Dibenzo[*a,h*]-anthracene | 1.54 (3.37) | 2.50 (0.83) | 5.29 (0.62) | 3.23 (0.62) | 1.95 (0.20) | 2.80 (1.50) | 0.56 (0.02) | 19.1 (0.26) | 146 (0.24) | 51.3 (2.68) | 4,220 (1.40) | 18,140 (3.38) | 2.0 (0.28) |
| Benzo[*g,h,i*]-perylene | 2.94 (6.43) | 3.77 (1.25) | 7.76 (0.90) | 3.57 (0.69) | 3.33 (0.34) | 5.43 (2.90) | 11.8 (0.41) | 291 (3.93) | 6,760 (11.33) | ND | 63,600 (21.07) | 110,000 (20.51) | ND |
| Indeno-[1,2,3,*c,d*]pyrene | 2.22 (4.86) | 2.96 (0.98) | 3.39 (0.39) | 2.94 (0.57) | 4.32 (0.44) | 4.70 (2.51) | 12.0 (0.42) | 393 (5.31) | 5,860 (9.83) | 22.0 (1.15) | 50,200 (16.63) | 119,000 (22.18) | 4.4 (0.63) |
| Sum | 45.7 | 301 | 860 | 518 | 987 | 187 | 2,847 | 7,404 | 59,642 | 1,912 | 301,896 | 536,441 | 703 |

ND, not detected. Values shown are in nanograms per gram, with percent shown in parentheses.

fly ash samples (Fig. 2D, Table 1) from an NMRF. With 1.6–3%, PCBs accounted for a higher percentage of total TEQ than in other sample types. Furthermore, relatively high PAH contents led to a larger difference between EROD-TEQ and chemical TEQ. Detailed analysis of PAHs showed a relative abundance of highly condensed PAHs such as benzo[*g,h,i*]perylene and indeno[1,2,3,*c,d*]pyrene in two samples and benzo[*k*]fluoranthene in a third sample (NMRI 1c).

A correlation analysis of the data from all samples revealed that the EROD-TEQ data were in reasonable correlation with the sum of PCDD/PCDF and dioxinlike PCB levels (Fig. 4A), showing a correlation coefficient of 0.75. When the same type of analysis was performed for the six MWI samples (Fig. 4B), an even better correlation was found ($r = 0.90$). The influence of total PAHs on the difference between chemical TEQ and EROD-TEQ is shown in Figure 3, suggesting a rough correlation between both.

In additional experiments, the contribution of PCDDs/PCDFs, dioxinlike PCBs, and PAHs to the overall EROD-inducing potency of a sample was estimated using three fractions enriched in these subtypes of contaminants. We found (Fig. 5A) that the PAH fraction of sample NMRF 1a made a major contribution to the total EROD-TEQ; the sum of EROD-TEQ of the individual fractions even exceeded the value for the raw extract (Fig. 2A). In contrast, the sum of EROD-TEQ of fractions of sample MWI 1/27-29 (Fig. 5B) was far below the EROD-TEQ of the raw extract (Fig. 2A), suggesting a loss of (unidentified) AhR agonists during the fractionation procedure.

## Discussion

This study was designed to compare results from the EROD bioassay in rat hepatocyte cultures to those from chemical analysis of PCDDs, PCDFs, and dioxinlike PCBs in fly ash extracts from various types of incinerators. The relevant emissions from incinerators are not necessarily identical to the fly ash samples collected from filters. However, such samples have been widely used as a surrogate for emissions (*25–27*).

Chemical analysis focused on 2,3,7,8-substituted PCDD/PCDF congeners and the dioxinlike PCBs attributed with TEFs by international working groups (*8,9*). The concentration of potent PCDDs/PCDFs and dioxinlike PCBs, expressed as chemical TEQ per gram fly ash, varied considerably between the samples, even samples from different incinerators of different design in the same plant showing up to 25-fold differences in PCDD/PCDF-TEQ and up to 91-fold differences in PCB-TEQ. A high variability in TEQ contents in fly ash samples was also reported by Sawyer et al. (*25*).

Interestingly, the levels of PCDDs/PCDFs and dioxinlike PCBs in MWI fly ash samples were in reasonably good correlation. This finding may indicate that the relative levels of PCDDs/PCDFs and dioxinlike PCBs are influenced by the same parameters of the waste incineration process

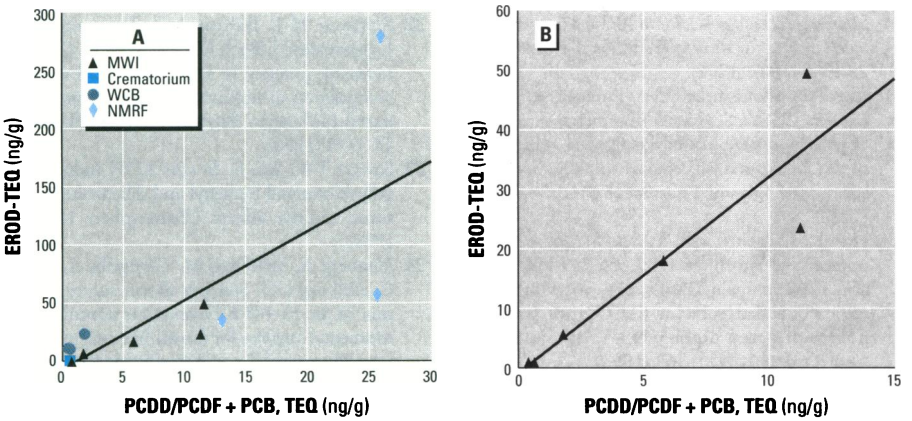


**Figure 4.** Linear correlation analysis between chemical toxic equivalents (TEQ) for potent polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDDs/PCDFs) and dioxinlike polychlorinated biphenyls (PCBs), and 7-ethoxyresorufin *O*-deethylase (EROD)-TEQ for (A) all incinerator fly ash examined including samples from municipal waste incinerators (MWI), a crematorium, wood combustors (WCB), and a noble metal recycling facility (NMRF) ($r = 0.75$); and (B) for extracts of MWI fly ash samples only ($r = 0.90$). EROD-TEQ values represent the means of four independent determinations using the median effective concentration of EROD induction in rat hepatocytes in primary culture.

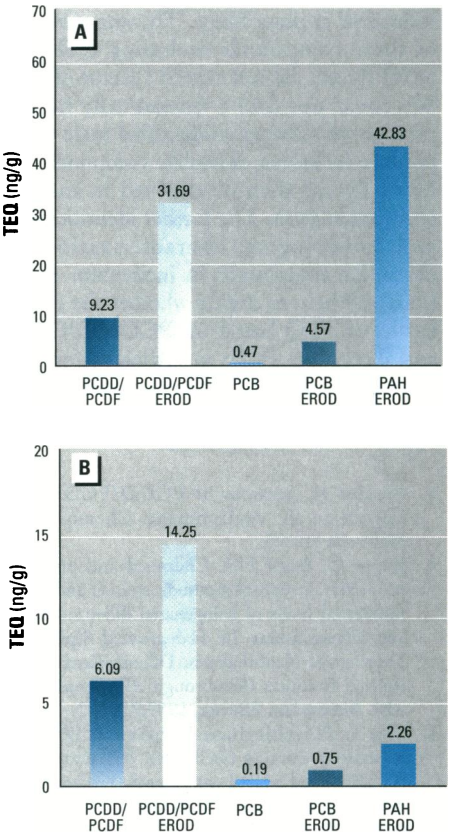


**Figure 5.** Chemical toxic equivalent (TEQ) concentrations and 7-ethoxyresorufin *O*-deethylase (EROD)-TEQ of fractions of potent polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDDs/PCDFs), dioxinlike polychlorinated biphenyls (PCBs), and 16 major polycyclic aromatic hydrocarbons (PAHs). (A) Sample NMRF 1a from a noble metal recycling facility (sum of PAHs = 10.1 µg/g). (B) Sample MWI 1/27-29 from a municipal waste incinerator (sum of PAHs = 0.7 µg/g) (see Fig. 2). Fly ash samples were prepared and fractionated, and potent PCDDs/PCDFs, dioxinlike PCBs, and PAHs were analyzed in the fractions as described in Methods. $TEQ_{EROD}$ values of the fractions were determined using the median effective concentration ($EC_{50}$) of EROD induction in the EROD bioassay. EROD-TEQ values represent the means of four independent determinations using the $EC_{50}$ of EROD induction in rat hepatocytes in primary culture.

such as type of burned material, temperature, oxygen supply, fly ash composition, etc., which is in agreement with a previous report by Schoonenboom et al. (*31*).

In the EROD bioassay, most MWI samples exhibited a two- to fivefold higher inducing potency than expected from the calculation of chemical TEQ. Because AhR agonists are also present among PAHs usually formed during incineration of organic material, a set of 16 major PAHs described by the EPA (*32*) was analyzed. In fact, the total amount of these PAHs was roughly correlated to the gap between EROD-TEQ and chemical TEQ, suggesting that PAHs play a certain role as EROD inducers in MWI samples.

A crematorium fly ash sample also fit into this picture, showing a twofold higher EROD-TEQ than expected, which was in agreement with the relative abundance of PAHs in this sample.

Fly ash samples from three WCB were found to contain EROD-TEQ in the same range as the MWI fly ash samples, in spite of the fact that PCDDs/PCDFs were in the lower range and dioxinlike PCBs were found in relatively low concentrations. A fly ash extract from an NMRF also showed an extraordinary wide gap between chemical TEQ for PCDDs/PCDFs plus dioxinlike PCBs and EROD-TEQ. Our data indicate that PAHs found at relatively high levels in WCB and NMRF fly ash samples play an important role in providing inducing equivalents, whereas no simple correlation between the sum of 16 PAHs and the chemical TEQ/EROD-TEQ gap was evident.

The outcome of the bioassay may be influenced by a number of factors other than the total amount of 16 PAHs. Because PAHs not analyzed in this study also have been shown to act as AhR agonists (*33*), an influence of these compounds cannot be ruled out. In addition, synergistic interactions of PAHs (*34*) and other dioxinlike compounds appear to be possible. However, previous investigations demonstrated that, in the bioassay, PCDDs do not interact synergistically (*19*) and that PCBs exhibit only a minor synergistic interaction (*21*). A synergistic enhancement of EROD induction between PAHs and PCDDs/PCDFs in the samples studied appears unlikely because a fractionation experiment with a PAH-rich sample (NMRF 1a) showed that the sum of individual contributions of subfractions enriched in PAHs, PCDDs/PCDFs, and dioxinlike PCBs to the EROD-TEQ was even higher than the value found with the raw extract (complete mixture). In another fractionation experiment with an MWI fly ash extract, however, the EROD-TEQ value of the PAH-enriched fraction only provided a minor contribution to the gap between EROD-TEQ and chemical TEQ, suggesting that other dioxinlike compounds such as certain polybrominated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls, polyhalogenated naphthalenes, biphenyl ethers, terphenyls, or PAHs not analyzed in this study play a major role in this sample. These compounds are either lost during the fractionation or act synergistically with other types of inducers in the unfractionated extract.

In conclusion, it is shown that fly ash samples from different types of thermal processes can differ widely in the TEQ level of PCDDs/PCDFs and dioxinlike PCBs and in the concentration of PAHs. The EROD bioassay was found to be suitable as an estimate for the level of PCDDs/PCDFs (and dioxinlike PCBs) in fly ash samples from MWI or from a crematorium containing relatively low levels of PAHs. Experimental EROD-TEQ of MWI fly ash extracts were about equal or approximately two- to fivefold higher than those calculated from chemical analysis, whereas a stronger overestimation was found for fly ash samples from wood burning or NMRF. These differences are partially due to PAHs present in these samples in very different patterns and concentrations. However, the presence of additional dioxinlike constituents leading to a measurable EROD

induction appears likely. The contribution of these compounds including PAHs in MWI fly ash did not exceed a factor of five. We could not find a dramatically higher presence of hitherto unidentified toxic compounds, including dioxinlike compounds in MWI fly ash than determined in routine chemical analysis. The further identification and toxicological characterization of dioxinlike compounds in incinerator emissions will help to decide whether the calculation of TEQ based on PCDDs/PCDFs and dioxinlike PCBs is sufficient for risk assessment and regulatory measurements.

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